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Light-Stimulated Degradation of an Unassembled Rieske FeS Protein by a Thylakoid-Bound Protease: The Possible Role of the FtsH Protease

Oren Ostersetzer and Zach Adam[1]

Department of Agricultural Botany, Hebrew University of Jerusalem, Rehovot 76100, Israel

Unassembled subunits of the cytochrome b_6f complex as well as components of other unassembled chloroplastic complexes are rapidly degraded within the organelle. However, the mechanisms involved in these proteolytic processes are obscure. When the Rieske FeS protein (RISP) is imported into isolated chloroplasts in vitro, some of the protein does not properly assemble with the cytochrome complex, as determined by its sensitivity to exogenous protease. When assayed in intact, lysed, or fractionated chloroplasts, the imported RISP was found to be sensitive to endogenous proteases as well. The activity responsible for degradation of the unassembled protein was localized to the thylakoid membrane and characterized as a metalloprotease requiring zinc ions for its activity. The degradation rate was stimulated by light, but no involvement of ATP or redox control was observed. Instead, when the RISP that was attached to thylakoid membranes was first illuminated on ice, degradation proceeded in either light or darkness at equal rates, suggesting a light-induced conformational change making the protein prone to degradation. Antibodies raised against native FtsH, a bacterial, membrane-bound, ATP-dependent, zinc-stimulated protease, effectively inhibited degradation of the unassembled RISP, suggesting a role for the chloroplastic FtsH in this process.

INTRODUCTION

The Rieske FeS protein (RISP), a component of the thylakoid-bound cytochrome (Cyt) b_6f complex, mediates photosynthetic electron transport between Cyt b_6 and Cyt *f* (reviewed in Cramer et al., 1996). Although it is still a matter of dispute whether the RISP is an intrinsic or extrinsic membrane protein (Breyton et al., 1994; Madueño et al., 1994; Cramer et al., 1996), it is widely accepted that the bulk of the protein, including the 2Fe-2S center that is active in redox reactions, faces the lumen. This topology is consistent with the RISP's role in shuttling electrons between the membrane-embedded heme group of Cyt b_6 and the one exposed to the lumen of Cyt *f*. The RISP is synthesized in the cytosol in a precursor form, with an N-terminal signal peptide that directs it to the chloroplast and mediates its post-translational translocation across the envelope (Madueño et al., 1992). During or shortly after import, this signal peptide is proteolytically removed, and the mature protein undergoes further sorting and assembly processes, leading to its final association with other subunits of the cytochrome complex in the thylakoid membrane. Although details of these processes are not yet known, the RISP is found transiently associated with stromal chaperones, such as Cpn60 and Hsp70, which are believed to be involved in keeping the protein in a conformation competent for integration into the membrane by a pathway that depends on the proton motive force (Madueño et al., 1993).

Because the cytochrome b_6f complex is composed of subunits encoded in the chloroplast (Cyt *f*, Cyt b_6, subunit IV, PetG, and PetL) and nucleus (RISP and PetM) (Haley and Bogorad, 1989; Schmidt and Malkin, 1993; Berthold et al., 1995; de Vitry et al., 1996; Takahashi et al., 1996), a tight cooperation between these two genomes is needed to ensure stoichiometric amounts of the different components. How this cross-talk between the two genomes is achieved is not yet clear, but proteolytic degradation is assumed to be involved in fine-tuning the amounts of the different subunits via degradation of unassembled subunits. The existence of proteolytic machinery capable of degrading subunits of the cytochrome complex can be inferred from experiments with a Lemna mutant. The results of these experiments showed that when the cytochrome complex is not fully assembled, its subunits are proteolytically degraded (Bruce and Malkin, 1991). In this case, a nuclear mutation affecting the level of RISP mRNA resulted in the inability to accumulate the other subunits of the complex, although their mRNA levels were normal. Similarly, deletion of the *petG* (Berthold et al., 1995) and *petL* (Takahashi et al., 1996) genes of Chlamydomonas resulted in reduced levels of other components of the cytochrome complex. Degradation of unassembled subunits of other proteins, due to either mutations or selective inhibition of synthesis of one

[1] To whom correspondence should be addressed. E-mail zach@agri.huji.ac.il; fax 972-8-946-7763.

subunit, has also been demonstrated (Schmidt and Mishkind, 1983; Leto et al., 1985; Avni et al., 1989).

Under in vitro conditions when the RISP is imported into isolated chloroplasts, only a certain proportion of the imported protein is correctly assembled with the cytochrome complex. This is indicated by the resistance of some of the protein to exogenously added protease, whereas the rest is sensitive to this treatment (Madueño et al., 1993, 1994). Because the proteolytic machinery involved in the degradation of unassembled subunits of the cytochrome complex in vivo has not yet been characterized, we decided to exploit the in vitro phenomenon to gain insight into the relevant proteolytic machinery. In this study, we demonstrate that the unassembled RISP is rapidly degraded by a mechanism that is stimulated by light but independent of ATP. The enzyme involved is a thylakoid-bound metalloprotease stimulated by zinc ions. This enzyme might be related to the family of FtsH proteases found in bacteria, mitochondria, and chloroplasts.

RESULTS

Stability of the Endogenous and Imported RISP

When the in vitro–synthesized wild-type RISP is imported into intact chloroplasts, it is correctly targeted to the thylakoid membrane and assembled with the cytochrome b_6f complex (Madueño et al., 1993, 1994). However, a certain proportion (40 to 80%) of the imported protein remains associated with the stromal side of the thylakoid membrane and does not assemble with the complex, as indicated by its sensitivity to protease treatment (Figure 1; Madueño et al., 1993, 1994). This idea is further supported by the observation that the endogenous RISP is much less sensitive to the protease treatment, as determined by the protein gel blot analysis presented in Figure 1. The susceptibility of the unassembled RISP to exogenous protease prompted us to test its stability in intact chloroplasts. To that end, we synthesized two mutants of RISP, in addition to the wild-type protein: the soluble mutant was targeted to the stroma (data not shown) due to the deletion of a hydrophobic sequence (designated Δ68-92), and the membrane-bound mutant contained a single amino acid substitution in the putative Fe-S center binding domain (designated C162S). The wild-type and mutant proteins were imported into intact chloroplasts, and after completion of the import reaction, the chloroplasts were subjected to further incubation in the light and in the presence of supplemented ATP.

PAGE analysis followed by autoradiography, as shown in Figure 2, revealed that the RISP and its two mutants are unstable, with half-lives of 60 to 90 min. In contrast, other imported proteins, such as the stromal ribulose-1,5-bisphosphate

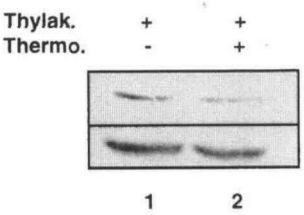

Figure 1. Resistance of the In Vitro–Imported and Endogenous RISP to Protease Treatment.

After import of the RISP, thylakoids (Thylak.) were isolated and treated with thermolysin (Thermo.). The amounts of labeled imported RISP and endogenous RISP remaining after this treatment were assayed by PAGE followed by autoradiography (top) or protein gel blot analysis (bottom), respectively.

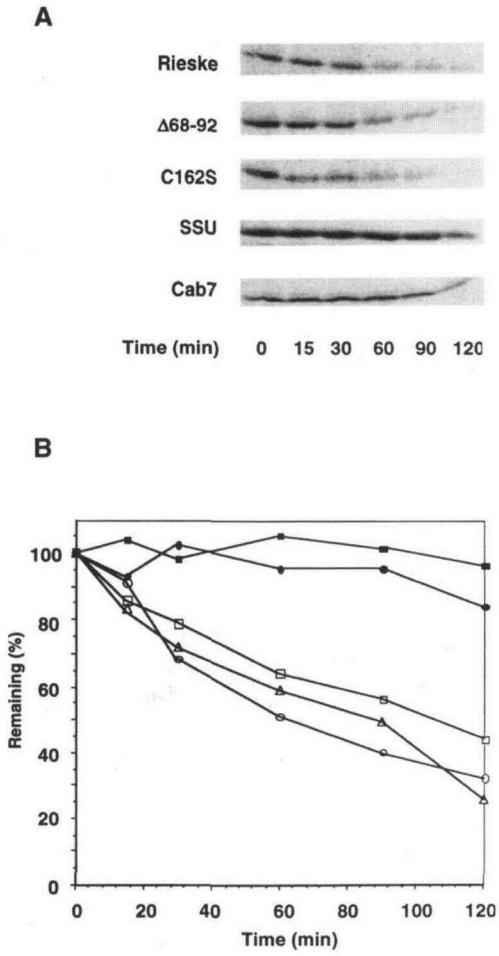

Figure 2. Stability of Imported Proteins in Intact Chloroplasts.

(A) Autoradiography representing postimport stability of the RISP, its two mutants (Δ68-92 and C162S), SSU, and Cab7.

(B) Quantification of bands in **(A)**: (●), Cab7; (■), SSU; (□), RISP; (△), Δ68-92; (○), C162S.

carboxylase small subunit (SSU) or a thylakoid-bound chlorophyll (Chl) *a/b* binding protein (Cab7), remained stable over a period of 2 hr (Figures 2A and 2B), as has been previously observed for some mutants of the SSU (Adam, 1995; Levy and Adam, 1995) and the lumenal protein OEE33 (Halperin and Adam, 1996). Thus, the in organello system mimicked the in vitro system in that the unassembled RISP was sensitive to endogenous or exogenous proteases, respectively. This observation enabled us to characterize further the degradation process of the unassembled RISP.

Localization of the RISP-Degrading Activity

To localize the proteolytic activity, we lysed chloroplasts immediately after the import reaction and fractionated them into thylakoids and stroma. We then compared the degradation rates of the imported RISP in lysates, isolated thylakoids, and thylakoids supplemented with stroma. The results, summarized in Figure 3A, demonstrate that degradation of the wild-type RISP in isolated thylakoids exhibited kinetics similar to that observed in lysates, and the addition of stromal extract to the thylakoids had no significant effect on degradation. Thus, it appears that the thylakoid-associated, unassembled RISP is degraded by a thylakoid protease, with no significant contribution of stromal proteases to the process. Similar results were obtained when degradation of the C162S mutant was monitored (data not shown). Surprisingly, whereas the stroma-localized mutant Δ68-92 was degraded in lysates, it was completely stable in the stroma, and only the addition of isolated thylakoids initiated its degradation (Figure 3B).

The observed degradation of the imported RISP in isolated thylakoids allowed us to test further the assembly state of the unstable protein. Because we observed two populations of the imported RISP, one sensitive and the other resistant to exogenous protease, we tested their respective sensitivities to the endogenous thylakoid-bound protease. After the import of the RISP into chloroplasts, we isolated thylakoids, treated them with thermolysin, and tested the stability of the thermolysin-insensitive population over time. In contrast to the entire population of the imported RISP, the protein resistant to thermolysin was stable during additional incubation (data not shown), supporting the notion that the unstable protein represents the unassembled RISP.

Effects of Light and Temperature on Degradation of the RISP

To characterize further the proteolytic mechanism responsible for the degradation of the unassembled RISP, we imported the radiolabeled protein into chloroplasts and conducted a chase incubation in the absence of added ATP in either the light or dark. In chloroplasts incubated in the dark, degradation was relatively slow, with ~80% of the protein remaining intact after 2 hr (Figure 4A). Incubation in the light greatly stimulated degradation. This positive effect of light on the degradation rate was also observed when using the two mutant proteins and isolated thylakoids rather

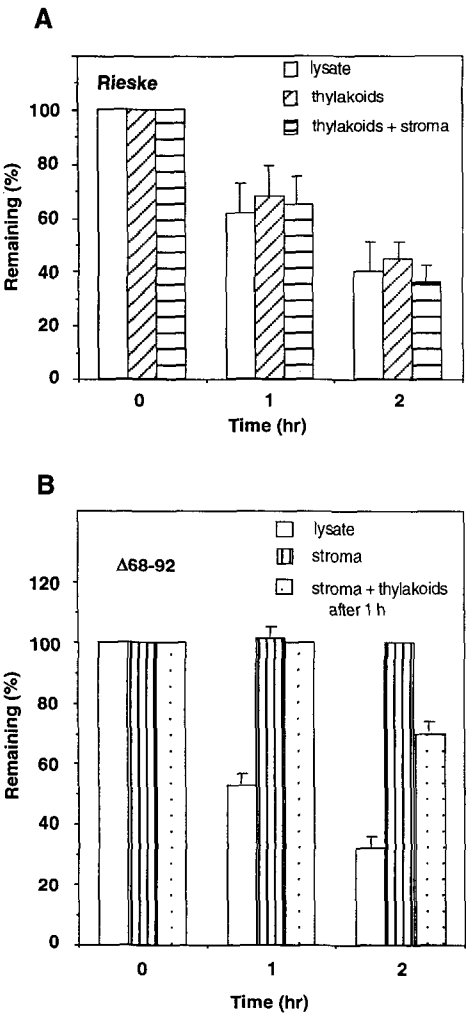

Figure 3. Stability of the Imported RISP and Its Soluble Mutant in Chloroplast Lysate and Subfractions.

(A) After import of the RISP, chloroplasts were either lysed or fractionated, and the amount of the remaining RISP in lysates, thylakoids, and thylakoids supplemented with stroma was determined. The results are means of three replicates, and the vertical bars indicate standard errors.

(B) After import of the soluble mutant, chloroplasts were either lysed or fractionated, and the amount of remaining soluble mutant remaining in the lysate or stroma was determined. In a third assay, after incubation of the stromal extract for 1 hr (h), isolated thylakoids were added. The results are means of three replicates, and the vertical bars indicate standard errors.

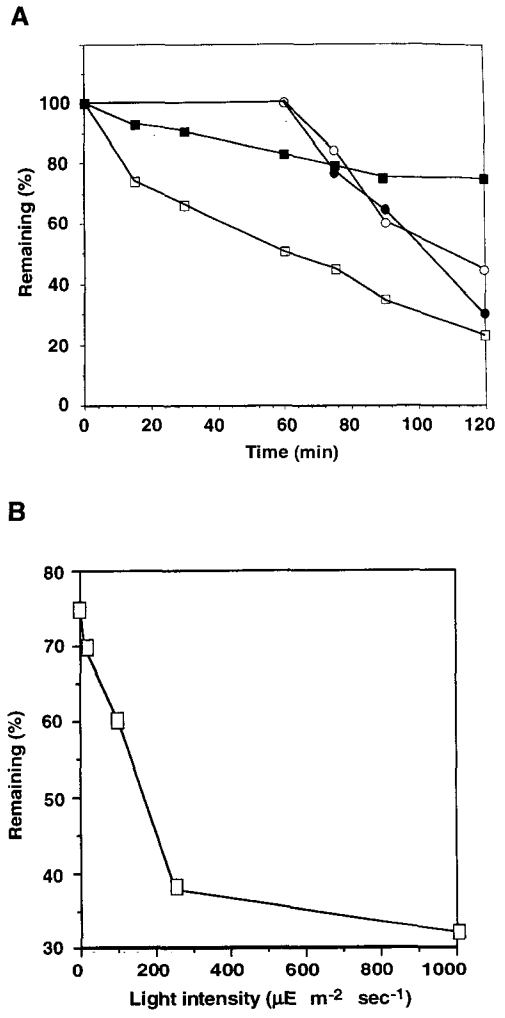

Figure 4. Effects of Light and Temperature on Degradation of the Imported RISP in Intact Chloroplasts.

(A) After import, chloroplasts were incubated at room temperature in either the light (□) or dark (■). At the time indicated, samples were taken, and the amount of the remaining RISP was determined. Alternatively, chloroplasts were kept on ice in the light for 1 hr, after which they were incubated at room temperature for another hour in either the light (○) or dark (●).

(B) After import, chloroplasts were incubated for 1 hr at room temperature at different light intensities, and the level of the remaining RISP was determined.

than intact chloroplasts as substrates (data not shown). Interestingly, the degradation rate was proportional to light intensity, with the response to light being almost linear in the range of 0 to 250 $\mu E\ m^{-2}\ sec^{-1}$ (Figure 4B).

To test whether light exerts its effect via ATP synthesis, which could be required for protein degradation, we added ATP to chloroplast lysates or washed thylakoids incubated in the dark. However, concentrations of 5 mM did not stimulate protein degradation (Table 1), eliminating the possibility that stimulation of degradation by light results from ATP synthesis. Consistent with this conclusion was the observation that the presence of ATP-consuming apyrase did not inhibit the light-stimulated degradation (Table 1). The direct involvement of redox components of the photosynthetic electron transport chain in controlling proteolytic activity could also be ruled out because various inhibitors of electron transport and ionophores, such as dichlorophenyl-dimethylurea, methyl viologen, and carbonyl cyanide *m*-chlorophenyl hydrazone, did not inhibit the light-stimulated degradation (data not shown). Sulfhydryl-modifying agents, such as β-mercaptoethanol, DTT, and 5,5′-dithio-*bis*-(2-nitrobenzoic acid), also had no effect on degradation.

Because the involvement of the photosynthetic machinery could not be demonstrated, we investigated whether light exerts its effect directly, either on the substrate protein or the protease itself. After import of the RISP into intact chloroplasts, they were incubated in the light on ice for 60 min. At the end of this incubation, no degradation was observed (Figure 4A). The chloroplasts were then incubated at 25°C in either the light or dark, and the level of the remaining RISP was determined. After this preexposure to light, degradation proceeded at equal rates in either the light or dark (Figure 4A). Thus, the actual degradation of the RISP appears to be able to proceed without light; however, light is a prerequisite for this process to occur. Currently, we cannot experimentally determine whether light is needed for activation of the protease itself or if it renders the protein substrate sensitive to proteolysis.

The Unassembled RISP Is Degraded by a Zinc-Stimulated Metalloprotease Related to FtsH

Figure 5 presents the effects of various protease inhibitors and ions on RISP degradation. We found that only EDTA

Table 1. Effect of ATP on RISP Degradation in Lysate and Thylakoids

Treatment	Remaining[a] (%)	ATP[b] (μM)
Lysate		
Light	37 ± 10	1.2
Dark	77 ± 12	
Dark + 5 mM ATP	81 ± 12	
Thylakoids		
Light	45 ± 11	ND[c]
Dark	77 ± 10	
Dark + 5 mM ATP	70 ± 8	
Light + apyrase	44 ± 6	ND

[a] Data represent means ± SE of four experiments.
[b] The ATP concentration was determined by the luciferin–luciferase assay.
[c] ND, not detectable.

and 1,10-phenanthroline, inhibitors of metalloproteases, inhibited degradation of the RISP (Figure 5A). Inhibitors of serine and cysteine proteases had no effect on the degradation. Thus, it is suggested that degradation of the unassembled RISP is performed by a membrane-bound metalloprotease. To test the effect of different divalent cations on degradation, thylakoid membranes isolated from chloroplasts after import were incubated with EDTA, washed, and resuspended in the presence of different cations. Among the ions tested, zinc was found most effective in stimulating the degradation of the unassembled protein (Figure 5B). Conducting the incubation in the presence of increasing concentrations of zinc revealed a K_m of 30 μM (data not shown).

We recently discovered that thylakoid membranes contain a homolog of the FtsH protease (Lindahl et al., 1996). In bacteria, the FtsH protease is characterized as a membrane-bound, ATP-dependent metalloprotease requiring zinc ions for its activity (Tomoyasu et al., 1995). Although we could not observe the stimulation of RISP degradation by ATP, localization of the activity to the membrane, together with the inhibition by metalloprotease inhibitors and stimulation by zinc, prompted us to test the possible involvement of the FtsH protease.

We first imported RISP into isolated chloroplasts. Thylakoid membranes were then isolated and incubated on ice with increasing amounts of antibodies raised against native FtsH from *Escherichia coli*. The samples were then transferred to 25°C in the light for an additional 60 min. At the end of this incubation, the amount of the remaining RISP was determined. As shown in Figures 6A and 6B, the antibody raised against native FtsH effectively inhibited RISP degradation, whereas incubation with equal amounts of preimmune serum had no such effect. Similarly, a specific antibody capable of recognizing only denatured but not native FtsH could not inhibit degradation of the RISP (data not shown).

Because these experiments were conducted with whole serum and not with an immunopurified antibody, we suspect that the observed inhibition could have resulted from the interaction of the antibody with another protease having an epitope similar to one of those found on native FtsH rather than reflecting inhibition of FtsH itself. Therefore, we compared the recognition patterns of both antibodies. Different patterns would indicate that the inhibition of RISP degradation could result from the interaction of the antibody with any of the cross-reacting proteins, whereas similar patterns would argue for FtsH's role in the degradation process. Therefore, thylakoids were resolved by SDS-PAGE and subjected to protein gel blot analysis. As shown in Figure 6C, the antibody capable of recognizing only denatured FtsH (lane 1) reacted primarily with a 78-kD species. A second band at ~40 kD was also observed. It was similar to the one seen after mild trypsin digestion (Lindahl et al., 1996), suggesting that this was a degradation product of full-length FtsH. The antibody generated against native FtsH from *E. coli* exhibited a similar pattern (Figure 6C, lane 2), although the relative intensities were different. These results suggest that inhibition of RISP degradation by the antibody against native FtsH probably was due to interaction with FtsH itself and not with a distantly related protein.

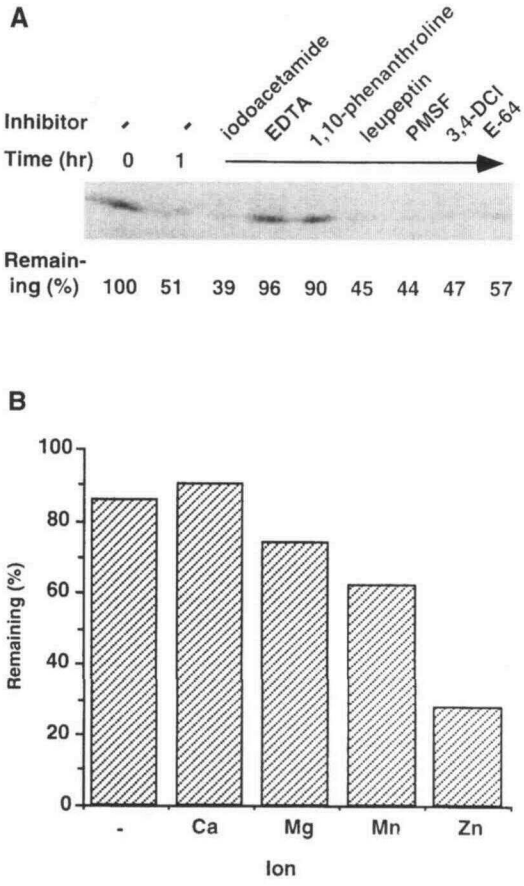

Figure 5. Effect of Protease Inhibitors and Divalent Cations on RISP Degradation.

(A) After import, thylakoid membranes were incubated in the presence of different protease inhibitors, and their effect on degradation was assayed. The remaining bands were quantified, and the results are indicated under the blot. PMSF, phenylmethylsulfonyl fluoride; 3,4-DCI, 3,4-dichloroisocoumarin; E-64, L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane, *N*-(*N*-[L-3-transcarboxyirane-2-carboxyl]-L-leucyl)-agmatine. (–) indicates no added inhibitor.

(B) After import, thylakoid membranes were isolated, incubated with EDTA, washed, and then resuspended in buffers containing different ions. (–) indicates no added ion.

DISCUSSION

The study of protein import, sorting, and assembly in chloroplasts has been greatly advanced by in organello and in vitro experiments using exogenously added protein substrates to intact chloroplasts or their subfractions. The added protein

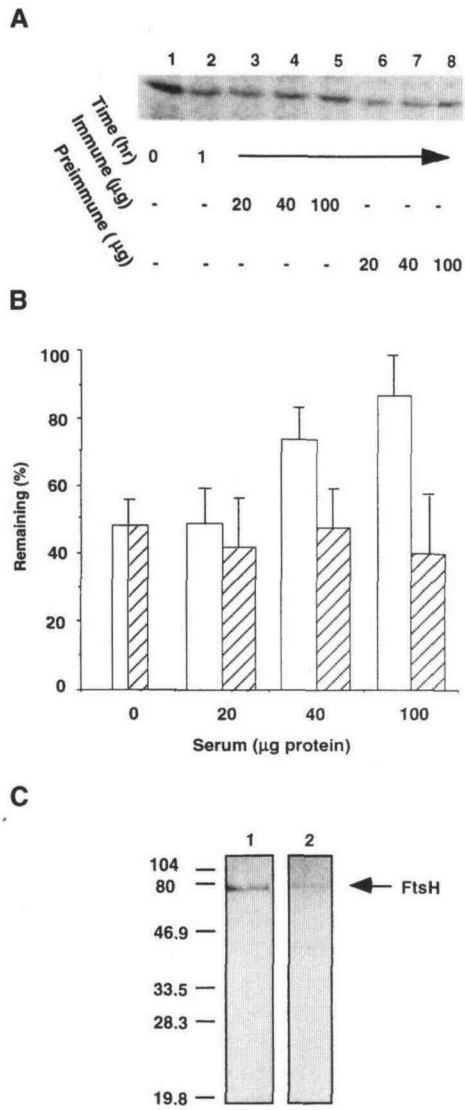

Figure 6. Effect of Antibodies Raised against the FtsH Protease on RISP Degradation.

(A) Thylakoid membranes were preincubated on ice with either specific antibodies raised against the native FtsH protease or preimmune serum before conducting an RISP stability assay. The dashes indicate no additions.

(B) Shown is the quantification of radioactive bands. Results are means ± SE (vertical bars) of four experiments. Open bars indicate immune serum; hatched bars represent preimmune serum.

(C) Shown are the results of protein gel blot analysis of thylakoid membranes with antibodies recognizing denatured (lane 1) and native (lane 2) FtsH. The migration of the molecular mass proteins (in kilodaltons) used as standards is indicated at left.

is assumed to behave like the protein in vivo, and this assumption is supported by the fact that exogenously added and endogenous proteins usually conform to the same criteria reflecting correct assembly. However, all imported proteins do not always behave similarly in such experiments, and some proteins remain unassembled, as seen here in Figure 1. We took advantage of this observation to study the fate of the unassembled RISP in the chloroplast. During the course of this study, however, we observed that the imported RISP is less stable than are other imported proteins. The reason for this difference is unclear, but it could well be that it reflects a difference in the turnover rates of the different proteins in the chloroplast. Those with relatively faster rates properly assemble with their complex counterparts and therefore remain stable, whereas those with slower turnover rates cannot exchange rapidly and, as a result, remain unassembled and prone to proteolysis. This explanation needs to be validated by a determination of turnover rates of different proteins in vivo.

The demonstration in this study that the unassembled RISP is unstable is quite similar to a previous observation of the rapid degradation of mistargeted OEE33 (Halperin and Adam, 1996). Interestingly, the half-lives of both proteins are similar, being on the order of 1 hr. However, the proteolytic machineries responsible for their degradation appear to be different. Whereas degradation of mistargeted OEE33 was mediated by a stromal enzyme, the unassembled RISP was degraded by a thylakoid-bound enzyme. Even a stroma-localized mutant of the RISP was degraded by the membrane-bound enzyme and was insensitive to stromal proteases (Figure 3B). For this observation, we have as yet no explanation. Energy requirements for both processes were also different: ATP was needed for OEE33 degradation, whereas RISP degradation appeared to proceed in the absence of ATP. Together with the fact that the two degradation processes were sensitive to different protease inhibitors, these results support the view that the chloroplast contains more than a single proteolytic machinery involved in its maintenance.

Of particular interest was the observation that RISP degradation is strongly stimulated by light. The lack of inhibition of degradation by a protonophore or apyrase and the lack of stimulation of degradation in the dark by ATP (Table 1) led us to the conclusion that the stimulative effect of light cannot be attributed to ATP synthesis. Redox control over the protease by components of the photosynthetic electron transport chain could also be ruled out because inhibitors of this process did not affect RISP degradation. Thus, it appears that light exerts its effect directly on either the substrate or the protease. This effect is most likely a physical one because it could be separated from the actual degradation. When membranes were first exposed to light on ice, no degradation was observed; only when the membranes were transferred to room temperature, in either the light or dark, did degradation proceed (Figure 4A). This situation is analogous to the degradation of the D1 protein of the photosystem II reaction center during photoinhibition: the D1 protein

can be degraded in the darkness after being primed for degradation in the light, suggesting that degradation occurs subsequent to photodamage rather than at the same time (Andersson et al., 1996).

What is the nature of this stimulative effect of light? Two possibilities can be considered. (1) The protease itself is activated by light, either directly or through another messenger. (2) The protein substrate changes its conformation to a protease-sensitive one either due to direct absorption of light energy or as a result of nonspecific oxidation. Because the unassembled RISP is bound to the thylakoid membrane containing photosynthetic pigments, excess light energy absorbed by these pigments, which is not dissipated by the photochemical process of photosynthesis, may affect the conformation of the unassembled RISP, leading to its increased sensitivity to proteolysis.

Because little is known about the identity of chloroplast proteases (Adam, 1996), tools for relating the observed RISP degradation to a known protease are rather limited. The characteristics of the activity responsible for RISP degradation are similar to those of the recently identified homolog of the FtsH protease in chloroplasts (Lindahl et al., 1996): both are bound to the thylakoid membrane, both are metalloproteases, and both are stimulated by zinc. Although we could not find any evidence of ATP's involvement in RISP degradation, a feature central to the activity of FtsH proteases (Pajic et al., 1994; Tomoyasu et al., 1995; Leonhard et al., 1996), we tested the possible involvement of FtsH in RISP degradation. Indeed, antibodies recognizing native FtsH from *E. coli* were effective in inhibiting the degradation of RISP (Figure 6), whereas other antibodies were not. Our suspicion that this inhibition could result from interactions with common epitopes shared by the FtsH antibody and another related protease were not supported by protein gel blot analysis using the inhibitory antibody. The pattern of recognition of this antibody was similar to that displayed by the noninhibitory antibody recognizing only denatured FtsH.

At least one other protein related to FtsH exists in plastids. A stromal factor involved in vesicle fusion and/or membrane protein translocation was recently cloned, and its sequence showed a significant homology to FtsH (Hugueney et al., 1995). However, its stromal location argues against its possible involvement in the degradation of the unassembled RISP. In this respect, it should also be noted that, similar to our observation, uncomplexed forms of SecY in *E. coli* were degraded by FtsH (Kihara et al., 1995).

If FtsH is indeed involved in the degradation of the unassembled RISP, how can the lack of an ATP effect be explained? ATP is not needed for the actual cleavage of peptide bonds by ATP-dependent proteases. Instead, it is believed to be required for unfolding of the protein substrate, making it more accessible to the active site of the protease. Thus, if the unassembled RISP is already unfolded, the action of the ATPase function of FtsH may not be essential. It has been previously shown that after import of the RISP into chloroplasts and before its translocation across the thylakoid membrane and assembly with the cytochrome b_6f complex, it is associated with the molecular chaperones Cpn60 and Hsp70 (Madueño et al., 1993). This association may keep the unassembled RISP in an extended conformation, rendering it accessible to the active site of FtsH. Validation of this hypothesis will require the isolation of authentic FtsH from thylakoid membranes.

Evidence for chaperone activity displayed by different ATP-dependent proteases has begun to accumulate recently. ClpA, the regulatory subunit of bacterial Clp protease, was able to replace DnaK and DnaJ in an in vitro chaperone assay (Wickner et al., 1994). Overproduction of a proteolytically inactive Lon protease in a yeast mutant could suppress developmental defects by serving a chaperone-like function in the assembly of mitochondrial protein complexes (Rep et al., 1996). The mitochondrial homologs of FtsH, YTA10 and YTA12, have independent of their proteolytic function a chaperone-like activity that is required for assembly of the membrane-bound ATP synthase (Arlt et al., 1996). Thus, in addition to being essential for proteolysis, the ATPase activity of ATP-dependent proteases appears to be required for assembly processes.

METHODS

Synthesis of Wild-Type and Mutant Precursor Proteins

The cDNA clone encoding the pea Rieske protein (Salter et al., 1992), cloned in pSP64, was generously donated by J. Gray (Cambridge University, Cambridge, UK). The 836-bp EcoRI-SspI fragment, containing the full-length cDNA, was subcloned into the EcoRI-EcoRV sites in pSP72 and used as a template for generating two Rieske protein mutants: one in which Cys-162 was replaced by Ser (C162S) and the other in which amino acid residues 68 to 92 were deleted (Δ68-92). Four oligonucleotides were synthesized and used for site-directed mutagenesis. They are 5′-GCCGTATGCACTCATCTCGGATCCGTCGTGCCG-3′, 5′-AGTAAGAGGAAGACCCCTCCAGGATCCGGTTCTTCA-3′, and their respective reverse oligonucleotides. These primers were used in four rounds of polymerase chain reaction. The first two oligonucleotides were used together with a T7 oligonucleotide, and the reverse oligonucleotides were used together with an SP6 oligonucleotide. The products of each two reactions were combined and amplified in a polymerase chain reaction with T7 and SP6 oligonucleotides to fuse the two products into a full-length clone, as described previously (Levy and Adam, 1995). The two products of these reactions were ligated into a pGEM-T vector (Promega) and subsequently subcloned into the EcoRI and BglII sites of pSP72. The mutations were verified by restriction analysis and DNA sequencing. The two mutant clones of the Rieske FeS protein (RISP) together with the wild-type clone were used as templates for the synthesis of the corresponding proteins. To synthesize the small subunit (SSU) of ribulose-1,5-bisphosphate carboxylase and the chlorophyll (Chl) *a/b* binding protein Cab7, we used pSP65-SSU (Levy and Adam, 1995) and pGEM4-Cab7 (Adam and Hoffman, 1993), respectively. Radiolabeled precursor proteins were synthesized in vitro in a coupled transcription–translation system containing wheat germ extract and SP6 RNA polymerase (Promega) in the presence of ^{35}S-Met.

Import and Stability of Imported Proteins

Intact chloroplasts, thylakoids, and stromal fractions were prepared from pea seedlings as described previously (Adam and Hoffman, 1993; Levy and Adam, 1995). Precursor proteins were imported by standard procedures (Adam and Hoffman, 1993), and the stability of the imported proteins in intact chloroplasts, lysed chloroplasts, or chloroplast subfractions was assayed as previously described (Adam, 1995; Levy and Adam, 1995; Halperin and Adam, 1996). The effect of the exogenous protease on the imported and endogenous RISP was assayed by incubating thylakoid membranes with 0.1 mg/mL thermolysin on ice for 30 min, and then samples were subjected to autoradiography or protein gel blot analysis, respectively. The effect of light intensities was assayed by placing the white-light source at decreasing distances from the samples with the temperature being kept at 25°C. The effect of different protease inhibitors was assayed at recommended concentrations (Beynon and Bond, 1989). To test the effect of ions, thylakoid membranes isolated from chloroplasts into which the RISP had been imported were first incubated with 10 mM EDTA for 30 min on ice. The membranes were then washed with 10 mM Hepes-KOH, pH 8.0, and resuspended to 2 μg of Chl/μL in the same buffer. Chloride salts of the tested ions were added to a final concentration of 1 mM before conducting the stability assay.

To assay the effect of different antibodies on RISP degradation, thylakoids were isolated from chloroplasts into which the RISP had been imported. They were then resuspended in 10 mM Hepes-KOH, pH 8.0, to 1 μg of Chl/μL and incubated on ice for 1 hr in the presence of increasing amounts (0 to 100 μg of protein) of different sera (see below). At the end of this incubation, the membranes were incubated for 1 hr at 25°C for the stability assay. Stability was determined by means of SDS-PAGE on 15% gels followed by the exposure of dried gels to PhosphorImager imaging plates (Fuji, Japan), and analysis and quantification of radioactive bands were determined by using a BAS100 image analyzer (Fuji).

Antibodies and Protein Gel Blot Analysis

To detect the endogenous RISP, we used an antibody generated against the maize protein (Voelker and Barkan, 1995), generously provided by A. Barkan (University of Oregon, Eugene). Two different antibodies raised against FtsH were generously provided by T. Ogura (Kumamoto University, Kumamoto, Japan). The serum of a guinea pig immunized with native FtsH from *Escherichia coli* recognized both the native and denatured proteins, whereas the serum of a rabbit immunized with a 16-mer synthetic peptide corresponding to a highly conserved region found in FtsH and related proteins (Tomoyasu et al., 1993) recognized only denatured FtsH. Protein gel blot analysis was performed by using standard methods (Harlow and Lane, 1988). The secondary antibodies used were alkaline phosphatase conjugated to goat anti-rabbit IgG for visualization of the rabbit antibodies and horseradish peroxidase conjugated to donkey anti-guinea pig IgG for detection of the guinea pig antibody.

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